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Flt3 Ligand Treatment Reverses Endotoxin Tolerance-Related Immunoparalysis

Maria Wysocka,*† Luis J. Montaner,† and Christopher L. Karp2‡

Endotoxin tolerance, the secondary blunting of a subset of microbial product-driven responses, is presumed to provide protection from pathological hyperactivation of the innate immune system during infection. However, endotoxin tolerance can itself be harmful. A significant percentage of sepsis survivors exhibit the phenotype of systemic endotoxin tolerance, a state termed immunoparalysis. Similar immune hyporeactivity, associated with an elevated risk of succumbing to bacterial superinfection, is also seen in the aftermath of major trauma, surgery, and burns. We recently demonstrated that in vivo endotoxin tolerance in murine models involves dendritic cell loss as well as alterations in the responsiveness of macrophages and remaining dendritic cells. Furthermore, the kinetics of recovery from immunoparalysis-associated inhibition of proinflammatory and immunoregulatory cytokine production directly parallels the kinetics of dendritic cell repopulation in these models. Given this, we examined whether recovery from immunoparalysis could be accelerated therapeutically with flt3 ligand, a growth factor that stimulates the differentiation and mobilization of dendritic cells. Notably, administration of flt3 ligand rapidly reverses immunoparalysis in vivo, accelerating and amplifying repopulation of tissues with proinflammatory and immunoregulatory cytokine-producing dendritic cells. The Journal of Immunology, 2005, 174: 7398–7402.

More than half a century after its initial description, the phenomenon of endotoxin tolerance continues to drive experiments and controversy (1). Although demonstrable both in vitro and in vivo, in purified cells as well as at the level of whole organisms, isolated monocyte/macrophages have provided prime targets of research into mechanism. It is clear in such cells that tolerance does not represent global inhibition of endotoxin-driven functions. Although LPS-induced production of diverse cytokines (e.g., TNF-α, IL-10, IL-12) is suppressed, LPS-induced production of other mediators (e.g., IL-1Ra) remains unaltered (2, 3). It is also evident that endotoxin tolerance is but a particular instance of a more general phenomenon of activation-induced monocyte/macrophage reprogramming. Similar effects are seen after priming with other TLR ligands or with mixtures of proinflammatory cytokines (4). The study of endotoxin tolerance has recently assumed new importance with the appreciation that a high percentage of survivors of sepsis (as well as of major trauma, surgery, and burns) exhibit prolonged systemic endotoxin tolerance, marked by suppression of stimulatable TNF-α and IL-12 production and low MHC class II expression by circulating monocytes (5–7). Patients exhibiting such immunoparalysis have a markedly elevated risk of succumbing to bacterial superinfection (5–7).

The molecular mechanisms underlying endotoxin tolerance remain controversial. Secretion of soluble mediators, changes in LPS receptor complex expression or function, alterations in TLR-driven signaling pathways or pathways of feedback inhibition, and/or primary effects on transcription, mRNA stability, and translational efficiency have all been implicated in one model or another (4, 8–11). One reason for a lack of consensus may be that the phenotype of endotoxin tolerance is quite complex, with distinct mechanisms being important for different aspects of the phenotype even in single cell types. For example: 1) although IL-10 and TGF-β are central to endotoxin-induced suppression of TNF-α in human monocytes, these cytokines appear to play no role in endotoxin tolerance-induced IL-12 suppression in such cells; and 2) although IFN-γ and GM-CSF are able to reverse endotoxin tolerance-related inhibition of TNF-α, they do not restore IL-12 production (2, 12, 13). In addition to the fact that the mechanisms responsible for endotoxin tolerance have remained obscure after reductive approaches involving single cell types in vitro, the analysis of such phenomena in vivo has inevitably revealed further layers of complexity (1, 14–16). Not surprisingly, the lack of a clear mechanistic understanding has hindered the development of therapeutic strategies for patients with immunological paralysis.

Although the experimental focus has remained largely on monocyte/macrophages, dendritic cells (DC) also appear to play an important role in endotoxin tolerance in vivo. LPS (and sepsis) not only induces maturation and migration of DC in vivo, but also triggers DC apoptosis (16–20). We recently demonstrated that endotoxin tolerance in in vivo mouse models involves marked DC loss, augmentation of macrophage numbers, and alterations in the responsiveness of remaining DC and macrophages (16). Notably, the kinetics of recovery from endotoxin tolerance-induced inhibition of TNF-α and IL-12 production directly parallels the kinetics of splenic DC repopulation in these models (16). Flt3 ligand (flt3L) is a growth factor that stimulates the differentiation and mobilization of DC, expanding DC subsets in a variety of tissues (21–24). Although we previously showed that flt3L treatment fails to provide prophylaxis against LPS-driven splenic DC apoptosis and endotoxin tolerance (16), a potential therapeutic role for flt3L in established LPS tolerance

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3 Abbreviations used in this paper: DC, dendritic cell; flt3L, flt3 ligand; μMT, C57BL/6-Igh-6m/Cgn mice.
remained to be examined. We thus examined whether flt3L-mediated generation and mobilization of naive DC, fully responsive to infectious stimuli, could accelerate recovery from immunoparalysis.

Materials and Methods

Mice

Female C57BL/6 mice were purchased from Harlan Sprague Dawley. Female C57BL/6-Igh-6tm/Cgn mice deficient in mature B cells (µMT) were purchased from The Jackson Laboratory. In all experiments, mice were used at 5–7 wk of age.

Reagents

LPS from *Escherichia coli* serotype 0127:B8 was purchased from Sigma-Aldrich. Human flt3L was kindly provided by Immunex (Amgen).

Induction of LPS tolerance in vivo; Flt3L treatment

Mice were injected i.p. with 20 µg of LPS or a similar volume (0.1 ml) of PBS (16). Daily i.p. treatment was begun 10 h later, with 10 µg of flt3L or a similar volume (0.1 ml) of PBS. At different times after priming, mice were challenged with i.v. injection of 100 µg of LPS. Serum was obtained from blood collected at 1 or 3 h after LPS challenge to assess TNF-α and IL-12 levels, respectively (16).

Analysis of LPS tolerance ex vivo

Single cell suspensions from the spleens of mice injected with PBS or LPS 3, 6, or 10 days earlier, treated or not with flt3L, were cultured in 24-well plates at a density of 3 × 10⁶ cells/well under LPS-free conditions in RPMI 1640 supplemented with 10% FCS (HyClone), L-glutamine, nonessential amino acids, 1 mM sodium pyruvate, 1 mM HEPES, 2-ME, and penicillin. After 48 h, splenic leukocytes were stimulated with LPS (1 µg/ml) for an additional 20 h, after which culture supernatants were harvested for cytokine analysis (16).

Cytokine assays

IL-12p70 and TNF-α were measured by a two-site radioimmunoassay as described previously (25) using the following Ab pairs: C18.2 and C17.15 (sensitivity 5–10 pg/ml) for IL-12 p70 (anti-IL-12 Abs were generated at The Wistar Institute); XT22 (generously provided by A. Sher, National Institute of Allergy and Infectious Diseases); and polyclonal anti-TNF-α Ab obtained from BD Pharmingen (sensitivity: 100 pg/ml) for TNF-α. rIL-12 (kindly provided by Genetic Institute, Boston, MA), and rTNF-α (from Hoffman La Roche) were used as standards.
Flow cytometry

For analysis of DC and macrophages in the spleen, leukocytes obtained from mechanically disrupted spleens were resuspended in PBS containing 3% FCS, and incubated with anti-CD11c-PE and anti-CD11b-FITC (1 mg/ml) (both obtained from BD Pharmingen) for 30 min on ice. Cells were washed twice and resuspended in 1% paraformaldehyde before analysis. Anti-hamster IgG-PE and anti-rat IgG2b-FITC Abs were used as negative controls (16).

For analysis of cytokine production by splenic DC and macrophages, splenic leukocytes were harvested 1 h after in vivo challenge with LPS (100 μg i.v.) and cultured for 5 h with 10 μg brefeldin A (Sigma-Aldrich). After surface staining, as above, with anti-CD11c-PE or anti-CD11b-FITC, intracellular staining was performed with anti-IL-12p40-PE, anti-TNF-α-PE, or isotype control-PE Abs (BD Pharmingen) using Fix & Perm reagents (Caltag Laboratories) following the manufacturer’s instructions.

Cells were analyzed with a FACSCalibur (BD Biosciences) flow cytometer. For each condition, 150,000 events were collected and analyzed using CellQuest software (BD Biosciences).

Statistical analysis

Comparisons were analyzed using the unpaired Student’s t test.

Results

Flt3L treatment accelerates and amplifies repopulation of splenic CD11c<sup>high</sup> DC, and amplifies splenic macrophage numbers, in endotoxin-tolerant mice

In vivo endotoxin tolerance involves rapid loss of splenic DC, as well as loss of responsiveness of splenic macrophages (whose numbers increase) and residual DC (16). As the kinetics of recovery from endotoxin tolerance-associated inhibition of cytokine production directly parallels the kinetics of splenic DC repopulation in these models (16), we examined whether recovery from endotoxin tolerance could be accelerated by administration of flt3L.

C57BL/6 mice were injected i.p. with 20 μg of E. coli LPS (or PBS as a control). Daily i.p. injections of flt3L (or PBS) were begun 10 h later, a time chosen as a mimic of the probable time course of therapeutic intervention for immunoparalysis in septic humans (i.e., after initial clinical stabilization). To allow flt3L therapy to have a detectable effect, spleens from treated mice were collected beginning 3 days (and 3 daily injections of flt3L) after LPS exposure, a time point marked by profound endotoxin tolerance in untreated mice (16). As shown in Fig. 1A, CD11c<sup>high</sup> DC constitute ~1.8% of splenic leukocytes in mock- (PBS-) primed mice. LPS treatment results in the virtual ablation of splenic CD11c<sup>high</sup> DC within 24 h after priming (16). In the absence of flt3L treatment, splenic CD11c<sup>high</sup> DC percentages do not recover until 6–10 days after systemic LPS exposure (Fig. 1A). As predicted, flt3L treatment led to acceleration and (augmentation) of splenic CD11c<sup>high</sup> DC repopulation, with such cells constituting 1.5%, 6.3%, and 16.5% of splenic leukocytes by 3, 6, and 10 days, respectively (Fig. 1A). Interestingly, the percentage of CD11c<sup>high</sup> DC in LPS-primed, flt3L-treated mice lagged slightly (and diminishingly) behind that of PBS-primed, flt3L-treated mice, consistent with the fact that LPS exposure led to splenic DC depletion before flt3L treatment. Indeed, the effects of LPS and flt3L on splenic DC kinetics were essentially additive, and hence likely independent (Fig. 1A).

As we and others have previously shown, unlike its effect on splenic DC, systemic exposure to LPS leads to a marked increase in the percentage of CD11b<sup>high</sup>CD11c<sup>−</sup> splenic (and peritoneal) macrophages (Fig. 1B) (16, 17). Despite this increase, such macrophages exhibit the phenotype of endotoxin tolerance, producing little in the way of proinflammatory or immunoregulatory cytokines (16). Mice exhibit significant up-regulation of bone marrow myeloid precursors throughout the course of endotoxin tolerance (15). Indeed, in the absence of flt3 treatment, splenic macrophage percentages continued to rise, reaching 8.5% (from a baseline in PBS-primed mice of 2.3%) 10 days after LPS exposure (Fig. 1B). Notably, flt3L treatment further augmented the splenic macrophage population during endotoxin tolerance, to 13.9% by 10 days after LPS priming (Fig. 1B). As with effects on splenic DC, the effects of LPS and flt3L on splenic macrophage percentages were essentially additive.

Flt3L treatment accelerates recovery of TNF-α- and IL-12-producing splenic DCs in endotoxin-tolerant mice

In parallel with accelerated splenic DC repopulation, flt3L treatment of LPS-primed mice led to acceleration of the recovery of splenic leukocyte cytokine production with stimulatable TNF-α production recovering to normal levels by 3 days, and stimulable IL-12 production recovering between 3 and 6 days after LPS exposure (recovery of both, in the absence of flt3L treatment, occurring between 6 and 9 days in this model), with continued increases thereafter (Fig. 2).

Consistent with our previously published data (16), CD11c<sup>high</sup> DC were the major producers of IL-12 and TNF-α in response to LPS, both in the presence and absence of flt3L treatment (Fig. 3). Indeed, no IL-12p40 expression above background by CD11b<sup>high</sup> splenic cells was seen (data not shown), whereas a low percentage of TNF-α-producing CD11b<sup>high</sup> cells was observed. Notably, the kinetics of flt3L-mediated reversal of endotoxin tolerance paralleled that of cytokine-producing CD11c<sup>high</sup> DC (Fig. 3). Although similar percentages of splenic DC were able to produce IL-12 and TNF-α in the absence or presence of flt3L treatment 6 d after LPS challenge, the dramatic increase in absolute numbers of DC due to flt3L treatment resulted in elevated percentages of such cytokine-producing cells as a function of total splenocyte numbers, percentages that directly mirrored the kinetics of cytokine production both ex vivo and in vivo in the presence (or absence) of flt3L treatment.

Flt3L treatment causes functional reversal of endotoxin tolerance in vivo

These data suggested likely in vivo efficacy for flt3L treatment. To exclude potential confounding effects introduced by the generation of Abs against LPS (so-called “late tolerance”; Abs to the O-Ags of LPS are found in the circulation of LPS-exposed mice as early as 4 days after exposure (1,16)), B cell-deficient (μMT) mice were...
used for in vivo assays. The effects of flt3L treatment on the kinetics of the splenic DC and macrophage compartments were indistinguishable in endotoxin-tolerant C57BL/6 mice (Fig. 1) and C57BL/6-μMT mice (data not shown). Notably, in parallel with splenic DC repopulation and splenic leukocyte cytokine production ex vivo, flt3L treatment led to accelerated recovery (and subsequent overshooting) of systemic TNF-α and IL-12 production in response to LPS challenge in endotoxin-tolerant mice (Fig. 4).

Congruent with data on the effects of flt3L treatment on DC kinetics in LPS- and PBS-primed mice (Fig. 1), flt3L treated, PBS-primed produced more systemic cytokines in response to LPS challenge than did flt3L-treated, LPS-primed: 2.2-fold more IL-12 and 4.5-fold more TNF-α on day 3 after priming; subsiding to 1.6- and 2.2-fold more, respectively, on day 6 after priming (data not shown).

**Discussion**

We demonstrate here that administration of flt3L rapidly reverses in vivo endotoxin tolerance, more than halving the time to recovery of proinflammatory and immunoregulatory cytokine production in this well-described mouse model of immunoparalysis. It appears likely that the mechanism of action of flt3L in this model is through the mobilization, differentiation, and tissue expansion of DC populations, i.e., not through reversal of endotoxin tolerance at the single cell level, but through functional reversal by way of acceleration and amplification of tissue DC replenishment.

In vivo, a primary role for effects on DC seems most likely as: 1) DC are the primary source of IL-12 among splenic leukocytes after LPS stimulation (Fig. 3) (16, 19); 2) the kinetics of recovery from endotoxin tolerance-associated inhibition of cytokine production directly parallels the kinetics of cytokine-producing splenic DC (Fig. 3); 3) serum cytokine (TNF-α, IL-12, and IL-10) levels parallel splenic leukocyte responses, but not tissue (peritoneal) macrophage responses, during endotoxin stimulation and tolerance (16); and 4) these findings are consistent with the known activities of flt3L. However, the cellular origin of cytokines measured in fluids such as serum remains, perforce, unclear. It thus possible that effects of flt3L on other cell populations such as blood monocytes or tissue macrophages (e.g., Kupffer cells (1)) may also play a role in flt3L-mediated reversal of endotoxin tolerance in vivo. The markedly elevated risk of bacterial superinfection and subsequent death in patients with immunoparalysis occurring in the aftermath of sepsis, major trauma, major surgery, and extensive burn injury has led to attempts at therapeutic reversal of this state of prolonged (weeks-long) immunological vulnerability. Research into immunoparalysis has largely focused on MHC class II expression and TNF-α production (5, 7, 13, 26), despite the likely important role of suppressed IL-12 production to the overall phenotype of immunoparalysis (6, 12, 27, 28). Both IFN-γ and GM-CSF have reached phase I trials for immunoparalysis in humans based on their ability to reverse endotoxin tolerance-related suppression of MHC class II expression and TNF-α production in vitro in...
FIGURE 4. Flt3L treatment accelerates reversal of immunoparalysis in vivo. C57BL/6-µMT mice were primed i.p. with 20 µg of E. coli LPS (or PBS as a control). Daily i.p. injections of flt3L (10 ng) or PBS were begun 10 h later. Mice were challenged i.v. with 100 µg of LPS 3 or 6 days after LPS priming. Serum was harvested from blood collected 1 h (TNF-α) or 3 h (IL-12) after challenge. Data represent means (±SD) of three mice per group from a single experiment that is representative of an experimental n = 3. * p = 0.0003, ‡ p = 0.002, ¶ p = 0.02, and δ p = 0.01, compared with PBS-treated mice.

human cells and in vivo in mice (5, 13, 29, 30). Notably, however, neither cytokine restores endotoxin tolerance-suppressed production of IL-12 in production in vitro (12). Whether GM-CSF is able to restore IL-12 production in vivo through up-regulation of myeloid cell precursors and differentiation (15). Such considerations suggest that the use of flt3L may have relative advantages as a therapeutic approach to immunoparalysis. The fact that administration of flt3L in the current studies led to levels of cytokine production that rapidly exceeded those at baseline suggests that: 1) flt3L therapy for immunoparalysis would need to be performed with care and careful monitoring; and 2) relatively short courses of therapy may suffice.

Provision of immunodulatory therapy for sepsis and septic shock has proven exceedingly difficult. In part, this results from the exigencies of timing: patients tend to present clinically with sepsis during experimental models. It may well be that the immunoparalysis that follows sepsis may provide a more tractable target for immunomodulation.

Disclosures
The authors have no financial conflict of interest.

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